DRAFT: August 31, 1994

DECISION DOCUMENT
TSCA SECTION 5(H)(4) EXEMPTION FOR
BACILLUS SUBTILIS

I. SUMMARY

Bacillus subtilis is an ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. B. subtilis produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. However, under most conditions, the organism is not biologically active but exists in the spore form. B. subtilis is considered a benign organism, as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low.

II. BACKGROUND

A. Introduction

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA is proposing expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that would be exempt meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected

product benefits, these exemptions will not present unreasonable risks.

B. Criteria for Minimizing Release from Manufacturing Facilities

The standards prescribed for the Tier I exemption require the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is proposing, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's proposed standards for minimizing emissions specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the bacteria used in fermentation processes are usually debilitated, either intentionally or through acclimation to industrial

fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is proposing that the number of microorganisms in fermentor gases be reduced by at least two logs prior to the gases being exhausted from the fermentor. EPA selected this number based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted in a gas from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also proposing that the requirements specify that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most individuals from the allergenic responses associated with

microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that entry be limited to essential personnel also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

C. Introduced Genetic Material Criteria

In order to qualify for either Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for metabolizing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the

complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms proposed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should not prevent most microorganisms from meeting the exemption criteria, since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only if the introduced genetic material has been altered or selected to enhance frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer

usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Bacillus subtilis, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of B. subtilis will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of B. subtilis, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of B. subtilis, and EPA's review of the conditions selected.

D. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Bacillus subtilis is discussed in the next unit.

III. EVALUATION OF BACILLUS SUBTILIS

A. History of Use

1. History of safe commercial use. Bacillus subtilis is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of enzymes, antibiotics, and other specialty chemicals. B. subtilis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and falls under the Class 1 Containment under the European Federal of Biotechnology guidelines.

2. Products subject to TSCA jurisdiction. To date, EPA has reviewed three premanufacture notices (PMNs) for strains of B. subtilis. One of the strains was modified for enhanced production of the enzyme a-amylase to be used primarily in production of ethanol for use as gasoline. Another strain was modified for enhanced production of a lipase enzyme for use in heavy duty detergents.

B. Identification of Microorganism

1. Classification. The genus Bacillus consists of a large number of diverse, rod-shaped gram positive bacteria which are capable of producing endospores that are resistant to adverse environmental conditions. B. subtilis is the type species of the genus. Historically, B. subtilis was a term given to all aerobic endospore-forming bacilli. Numerous species that appeared in the early literature as B. subtilis have since been designated as separate Bacillus species. B. subtilis can be distinguished from closely related Bacillus species by the use of API diagnostic test kits or pyrolysis gas-liquid chromatography. Because of changes in the classification of the genus and recent developments in methods of taxonomic identification, older strains may not actually be B. subtilis under present-day definitions.

2. Related taxa of concern. B. subtilis is part of the same large cluster of bacilli which includes pathogenic or opportunistic Bacillus species. This includes the B. cereus/anthracis/thuringiensis/mycoides group whose members are mammalian and insect pathogens and food poisoning agents. However, B. subtilis is distinguishable from the pathogenic bacilli as well as from the more closely related bacilli.

C. Risk Summary

1. Studies regarding potential for adverse effects. B. subtilis is not a frank human pathogen, but has been isolated from human infections. However, the literature suggests that before infection can occur, there must be immunosuppression of the host followed by inoculation in high numbers of the microorganism. B. subtilis does not produce significant quantities of extracellular enzymes or toxins and is generally considered to have a low degree of virulence. B. subtilis does produce the extracellular enzyme subtilisin that has been reported to cause allergic or hypersensitivity reactions in individuals repeatedly exposed to it.

The literature also indicates that ecological hazards associated with the use of B. subtilis are low. While there are reports suggesting that B. subtilis is a cause of abortion in livestock, Koch's postulates have not been satisfied in demonstrating that this microorganism was the causal agent. The association of B. subtilis with livestock abortions is quite low compared to the total number of livestock abortions caused by microorganisms. B. subtilis is not considered a plant pathogen.

2. Studies regarding survival in the environment. B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active, due to the various enzymes produced by members of the species. Unless a soil has been recently amended with organic matter providing readily utilizable nutrients, the bacilli exist in the endospore stage.

IV. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. Bacillus subtilis is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. In addition to assessing the risk of B. subtilis, EPA has developed criteria limiting the potential for transfer of and expression of toxin sequences, and the conditions of use specified in the exemption are met (Tier I) or will be reviewed by EPA to ensure adequate risk reduction (Tier II). EPA requirements for minimizing numbers of viable microorganisms emitted are within standard operating procedures for the industry, and both the procedures and the structures specified in the exemption are the type industry uses to protect their products from contamination.

The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

V. RECOMMENDATION AND RATIONALE

A. Recommendation: Strains which meet the present day classification as Bacillus subtilis are recommended for the section 5(h)(4) exemption.

B. Rationale

1. Risks from use of the recipient microorganism B. subtilis are low. B. subtilis is ubiquitous in the environment and the releases expected from fermentation facilities will not significantly increase populations of this microorganism in the environment. Although the possibility of human infection by B. subtilis is not non-existent, it is low in the industrial setting, because it occurs primarily in highly immunocompromised individuals. In the industrial setting with the use of proper safety precautions, good laboratory practices, and proper protective clothing and eyewear, the potential for infection of workers should be quite low. The only human health concern for workers in the fermentation facility is the potential for allergic reactions with chronic exposure to subtilisin. OSHA has established an exposure limit for subtilisin which must be met in the industrial setting. Although B. subtilis may be associated with livestock abortions, the use of this microorganism in fermentation facilities will not substantially increase the frequency of this occurrence.

2. Use of recombinant strains of B. subtilis which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. While not completely innocuous, B. subtilis presents low risk of adverse effects to human health or the environment. Because of the change in classification, older industrial strains of B. subtilis may not meet the present-day designation. As part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using B. subtilis. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These

expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption. When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

REQUEST FOR COMMENTS

The Risk Assessment to support the proposal of Bacillus subtilis as a candidate for the TSCA section 5(h)(4) tiered exemption recommends that only asporogenic strains with a sporulation deficiency of at least 10^{-7} be eligible for the exemption. However, this Decision Document recommends all strains of Bacillus subtilis for this exemption. The recipient microorganism B. subtilis was found to have little potential for adverse effects. The probability is low that the insertion of genetic material meeting EPA's criteria into such a microorganism will change its behavior so that it would acquire the potential for causing adverse effects. Therefore, there should be no need to restrict this exemption to asporogenic strains.

However, because there is a discrepancy in the recommendations of the Risk Assessment and the Decision Document, EPA requests comment on whether its current recommendation of all strains of B. subtilis as eligible for this exemption is appropriate or should be modified to limit the exemption only to asporogenic strains.

INTEGRATED RISK ASSESSMENT OF BACILLUS SUBTILIS

I. INTRODUCTION

Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. However, under most conditions the organism is not biologically active but exists in the spore form (Alexander, 1977). *B. subtilis* is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low.

History of Commercial Use and Products Subject to TSCA Jurisdiction

B. subtilis is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids. TSCA uses of proteases include cleaning aids in detergents and dehairing and batting in the leather industry. TSCA uses of amylases include desizing of textiles and starch modification for sizing of paper (Erikson, 1976).

The Agency has reviewed, under TSCA, three PMNs of genetically modified *B. subtilis* for production of a protease (P87-1030), alpha-amylase (P89-227), and lipase (P91-1154). EPA found that there were no unreasonable risks associated with the use of these recombinant strains for enzyme production in fermentation facilities.

II. IDENTIFICATION AND TAXONOMY

A. Overview

B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active due to the various enzymes produced by members of the species. Although

the actual numbers in existence in the environment for this species has not been determined, bacilli occur at population levels of 10^6 to 10^7 per gram of soil (Alexander, 1977). However, unless a soil has been recently amended with organic matter providing readily utilizable nutrients, the bacilli exist in the endospore stage. It is thought that 60 to 100% of soil bacilli populations exist in the inactive spore state (Alexander, 1977). Like most members of the genus, *B. subtilis* is aerobic, except in the presence of glucose and nitrate, some anaerobic growth can occur (Claus and Berkeley, 1986).

B. Taxonomy and Characterization

The genus *Bacillus* consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria that are motile by peritrichous flagella and are aerobic. Members of the genus are capable of producing endospores that are highly resistant to unfavorable environment conditions (Claus and Berkeley, 1986). The genus consists of a diverse group of organisms as evidenced by the wide range of DNA base ratios of approximately 32 to 69 mol% G + C (Claus and Berkeley, 1986), which is far wider than that usually considered reasonable for a genus (Norris et al., 1981).

B. subtilis is the type species of the genus. Historically, prior to the monographs of Smith in 1946 and 1952, *B. subtilis* was a term given to all aerobic endospore-forming bacilli (Logan, 1988). Numerous species that appeared in the early literature are no longer recognized as official species. Former species designations that are now considered to be members of the species *B. subtilis* include *B. aterrimus, B. mesentericus, B. niger, B. panis, B. vulgarus, B. nigrificans,* and *B. natto* (Gibson, 1944 and Smith et al., 1946 as cited by Gordon, 1973). Although in the past it has been designated as a separate species, the latest edition of Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) listed *B. amyloliquefaciens* as a member of the species *B. subtilis*. However, recently it has again achieved the status of a separate species (Priest et al., 1987).

The *Bacillus* species *subtilis, licheniformis,* and *pumilus* are closely related and there has been difficulty distinguishing among the three species that historically were grouped together as the subtilis-group or subtilis-spectrum (Gordon, 1973). These three species clustered together (78%) in the "*subtilis*" group in a numerical classification based on 118 unit characteristics of 368 strains of *Bacillus* (Priest et al., 1988). However, this major cluster contained four subclusters that could be identified as *B. subtilis, B. licheniformis, B. pumulis,* and *B. amyloliquefaciens*. Recent data in the literature have suggested

that it is possible to differentiate *B. subtilis* from *B. licheniformis* and *B. pumulis* by the use of pyrolysis-gas chromatography (O'Donnell et al., 1980) or by the use of API tests (Logan and Berkeley, 1981). In addition, *B. subtilis* and *B. amyloliquefaciens* show little DNA sequence homology to each other (Seki et al., 1975; Priest, 1981) and can also be distinguished from each other by pyrolysis-gas chromatography (O'Donnell et al., 1980) and by a few phenotypic properties including the production of acid from lactose (Priest et al., 1987).

In conclusion, it appears that *B. subtilis* can be distinguished from other closely related species. However, because of changes in the classification of the genus, and the recent development of new methods for taxonomic purposes, older strains may not actually be *B. subtilis* under present-day definitions.

C. Related Species of Concern

There are several species of the genus that are known pathogens. These include *B. anthracis* which is pathogenic to humans and other animals, and *B. cereus* which is a common cause of food poisoning (Claus and Berkeley, 1986; Norris et al., 1981). *B. thuringiensis, B. larvae, B. lentimorbus, B. popilliae*, and some strains of *B. sphaericus* are pathogenic to certain insects. Other species in the genus are considered "opportunistic pathogens".

In a numerical classification using 118 characteristics of 368 species of *Bacillus*, the species *B. thuringiensis, B. cereus*, and *B. mycoides* clustered together at 89 - 92% similarity (Priest et al., 1988). The *B. subtilis* group joined the *B. cereus* group at 72% relatedness. There is no difficulty in distinguishing between the toxin-producing strains of *Bacillus* and *B. subtilis*.

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Colonization

B. subtilis is widely distributed throughout the environment, particularly in soil, air, and decomposing plant residue. It has shown a capacity to grow over a wide range of temperatures including that of the human body (Claus and Berkeley, 1986). However, *B. subtilis* does not appear to have any specialized attachment mechanisms typically found in organisms capable of colonizing humans (Edberg, 1991). Given its

ubiquity in nature and the environmental conditions under which it is capable of surviving, *B. subtilis* could be expected to temporarily inhabit the skin and gastrointestinal tract of humans, but it is doubtful that this organism would colonize other sites in the human body (Edberg, 1991).

2. Gene Transfer

The transfer of gene sequences between strains of *B. subtilis* has been demonstrated when the strains were grown together in soil (Graham and Istock, 1979). In addition, Klier et al. (1983) demonstrated the ability of *B. subtilis* and *B. thuringiensis* to exchange high frequency transfer plasmids. Other studies have shown that *B. subtilis* has the ability to express and secrete toxins or components of the toxins that were acquired from other microorganisms through such transfers of genetic material. *B. subtilis* expressed subunits of toxins from *Bordatella pertussis* (Saris et al., 1990a, 1990b), as well as subunits of diphtheria toxin (Hemila et al., 1989) and pneumolysin A pneumococcal toxin (Taira et al., 1989). Although *B. subtilis* does not appear to possess indigenous virulence factor genes, it is theoretically possible that it may acquire such genes from other bacteria, particularly from closely related bacteria within the genus.

3. Toxin Production

A review of the literature by Edberg (1991) failed to reveal the production of toxins by *B. subtilis*. Although it has been associated with outbreaks of food poisoning (Gilbert et al., 1981 and Kramer et al., 1982 as cited by Logan, 1988), the exact nature of its involvement has not been established. *B. subtilis*, like other closely related species in the genus, *B. licheniformis, B. pumulis,* and *B. megaterium*, have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecithinase production and human disease in *B. subtilis*.

B. subtilis does produce an extracellular toxin known as subtilisin. Although subtilisin has very low toxigenic properties (Gill, 1982), this proteinaceous compound is capable of causing allergic reactions in individuals who are repeatedly exposed to it (Edberg, 1991). Sensitization of workers to subtilisin may be a problem in fermentation facilities where exposure to high concentration of this compound may occur. Exposure limits to subtilisin are regulated by Occupational Safety and Health Administration (OSHA) (29 CFR 1900, et seq.)

4. Measure of the Degree of Virulence

B. subtilis appears to have a low degree of virulence to humans. It does not produce significant quantities of extracellular enzymes or possess other virulence factors that would predispose it to cause infection (Edberg, 1991). There are a number of reports where *B. subtilis* has been isolated from human infections. Earlier literature contains references to infections caused by *B. subtilis*. However, as previously stated, the term *B. subtilis* was synonymous for any aerobic sporeforming bacilli, and quite possibly, many of these infections were associated with *B. cereus*. In a recent British review article, Logan (1988) cites more recent cases of *B. subtilis* infections in which identification of the bacterium appeared reliable. Infections include a case of endocarditis in a drug abuse patient; fatal pneumonia and bacteremia in three leukemic patients; septicemia in a patient with breast cancer; and infection of a necrotic axillary tumor in another breast cancer patient. Isolation of *B. subtilis* was also made from surgical wound-drainage sites, from a subphrenic abscess from a breast prosthesis, and from two ventriculo-atrial shunt infections (as cited by Logan, 1988).

Reviews of *Bacillus* infections from several major hospitals suggest that *B. subtilis* is an organism with low virulence. Idhe and Armstrong (1973) reported that *Bacillus* infections were encountered only twelve times over a 6-1/2 year period. Species identification of these *Bacillus* infections was not made. In another hospital study over a 6-yr. period, only two of the 24 cases of bacteremia caused by *Bacillus* (of a total of 1,038 cases) were due to *B. subtilis* (as cited by Edberg, 1991). Many of these patients were immunocompromised or had long term indwelling foreign bodies such as a Hickman catheter.

B. subtilis has also been implicated in several cases of food poisoning (Gilbert et al., 1981 and Kramer et al., 1982 as cited by Logan, 1988).

As previously mentioned, *B. subtilis* produces a number of enzymes, including subtilisin, for use in laundry detergent products. There have been a number of cases of allergic or hypersensitivity reactions, including dermatitis and respiratory distress after the use of these laundry products (Norris et al., 1981).

5. Conclusions

B. subtilis is not a human pathogen, nor is it toxigenic like some other members of the genus. The virulence

characteristics of the microorganism are low. According to Edberg (1991) either the number of microorganisms challenging the individual must be very high or the immune status of the individual very low in order for infection with *B. subtilis* to occur.

B. Environmental Hazards

1. Hazards to Animals

B. subtilis has been isolated from a number of cases of bovine and ovine abortions, however, the microorganism has never been identified as the causal agent (Logan, 1988). Reports on association of *B. subtilis* with livestock abortions are fairly rare, and of much lower frequency than with other *Bacillus* species, which are rare compared to all other microorganisms, especially viruses and fungi. *B. subtilis* has also been reported in 17 cases of bovine mastitis in which it was thought to be the causal agent (Fossum et al., 1986). However, the limited number of cases of mastitis associated with *B. subtilis* also is rare compared to mastitis caused by other microorganisms.

B. subtilis has also been shown to be capable of infecting and causing mortality of the 2nd instar larvae of the mosquito, *Anophelis culicifacies*, which is the primary insect vector of malaria in central India (Gupta and Vyas, 1989). *B. subtilis* was being investigated for use as a biocontrol agent in this study.

2. Hazards to Plants

B. subtilis is not considered to be a plant pathogen (7 CFR 330, et seq.; Claus and Berkeley, 1986). However, there are several reports in the literature that associate *B. subtilis* with certain plant diseases. Kararah et al. (1985) produced soft rot of garlic cloves by injecting *B. subtilis* into them. Bergey's Manual of Systematic Bacteriology notes that pectin and polysaccharides of plant tissues can be decomposed by *B. subtilis* and that this microorganism can cause soft rot of potato tubers (Claus and Berkeley, 1986). There are several abstracts obtained in a literature review that suggests that *B. subtilis* may cause other plant diseases, however, no more information was obtainable. One abstract reported that *B. subtilis* was the cause of a broad open cancer ulcera on Norway maples in forests in the Urals (Yakovleva et al., 1990). Another reported that an organism tentatively identified as *B. subtilis* was consistently isolated from glasswort (*Salicornia*) plants suffering from a soft-rot disease (Stanghellini and Rasmussen, 1989).

3. Hazards to Other Microorganisms

B. subtilis has been shown to produce a wide variety of antibacterial and antifungal compounds (Katz and Demain, 1977; Korzybski et al., 1978). It produces novel antibiotics such as difficidin and oxydifficidin that have activity against a wide spectrum of aerobic and anaerobic bacteria (Zimmerman et al., 1987) as well as more common antibiotics such as bacitracin, bacillin, and bacillomycin B (Parry et al., 1983). The use of *B. subtilis* as a biocontrol agent of fungal plant pathogens is being investigated because of the effects of antifungal compounds on *Monilinia fructicola* (McKeen et al., 1986), *Aspergillus flavus* and *A. parasiticus* (Kimura and Hirano, 1988), and *Rhizoctonia* (Loeffler et al., 1986).

Although *B. subtilis* produces a variety of antibiotic compounds in culture media, the importance of antibiotic production in the environment is unknown (Alexander, 1977).

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

B. subtilis is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986). This microorganism also falls under the Class 1 Containment under the European Federation of Biotechnology guidelines (Frommer et al., 1989).

The potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the

potential for worker exposure was considered to be potentially greatest, ie. near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

B. subtilis is a common saprophytic inhabitant of soils and is thought to contribute to nutrient cycling due to the variety of proteases and other enzymes members of the species are capable of producing. Growth normally occurs under aerobic conditions, but in complex media in the presence of nitrate, anaerobic growth can occur (Claus and Berkeley, 1986). Under adverse environmental conditions, *B. subtilis* produces endospores that are resistant to heat and desiccation (Claus and Berkeley, 1986). Specific data comparing the survivability of industrial and wild-type strains of *B. subtilis* were not available in the existing literature. However, the ability of *B. subtilis* to produce highly resistant spores and grow under a wide range of conditions indicates that released strains are likely to survive outside of containment.

2. Releases

Estimates of the number of *B. subtilis* organisms released per production batch are tabulated in Table 1. All calculations are based on use of asporogenic strains with a sporulation deficiency of 10^{-7}. The minimally controlled scenario assumes no treatment of the fermentor off-gas and assumes 100-fold (2 log) reduction of the maximum cell density of the fermentation broth

resulting from inactivation (Reilly, 1991). The containment criteria required for the full exemption scenario assume the use of in-line filters to treat vent gases and a 99% removal efficiency under normal operating conditions. They also assume an overall 6-log reduction relative to the maximum cell density of the fermentation broth resulting from inactivation steps (Reilly, 1991).

TABLE 1. Estimated Number of Viable Bacillus subtilis Organisms Per Production Batch

Release Media	Minimally Controlled (cfu/day)	Full Exemption (cfu/day)	Release (days/year)
Air Vents	$2x10^{8}$ - $1x10^{11}$	$2x10^{6}$ - $1x10^{9}$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{13}$	$7x10^{9}$	90
Soil/Landfill	$7x10^{15}$	$7x10^{11}$	90

Source: Reilly, 1991

In addition to the releases tabulated in Table 1, spores would be released at a rate of 1.7×10^{10} spores/day in solid wastes and 2×10^{8} spores/day in aqueous wastes (Reilly, 1991). These are "worst-case" estimates which assume that the inactivation procedure against spores is ineffective and the separation efficiency for the rotary drum filter is 99 percent.

3. Air

Specific data which indicate the survivability of *B. subtilis* in the atmosphere after release are currently unavailable. However, its ability to survive in a broad habitat range and produce spores suggests that this organism would be likely to survive after release. As with naturally-occurring strains, human exposure may occur via inhalation as the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Air releases from fermentor off-gas could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from this source, the sector averaging form of the Gaussian algorithm described in Turner (1970) was used. For purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by additional treatment of off-gases, potential human

inhalation dose rates are estimated to range from 3.0×10^3 to 1.5×10^6 cfu/year for minimally controlled systems and 3.0×10^1 to 1.5×10^4 cfu/year for systems with full exemptions. It should be noted that these estimates represent hypothetical exposures under reasonable worst case conditions (Versar, 1992).

4. Water

The concentrations of *B. subtilis* in surface water were estimated using stream flow values for water bodies receiving process wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release data (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/l). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers facilities that have a NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10-year period. The use of this methodology to estimate concentrations of *B. subtilis* in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process. Estimated concentrations of *B. subtilis* in surface water for minimally controlled and full exemption scenarios are tabulated in Table 2 (Versar, 1992).

TABLE 2. Bacillus subtilis Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	Q710	Mean	Q710
Minimally Controlled				
10th Percentile	156	5.60	4.5×10^{5}	1.25×10^{7}
50th Percentile	768	68.13	9.11×10^{4}	1.03×10^{6}
Full Exemption				
10th Percentile	156	5.60	4.5×10^{1}	1.25×10^{3}
50th Percentile	768	68.13	9.11×10^{0}	1.03×10^{2}

*MLD = million liters per day
Source: Versar, 1992

The concentrations of *B. subtilis* spores in surface water were also estimated using the methodology and assumptions described above. Estimated concentrations of *B. subtilis* spores in surface water are tabulated in Table 3.

TABLE 3. Concentrations of Bacillus subtilis spores in surface water

Flow	Spores/l	
	Mean	7Q10
l0th Percentile	1.28×10^{0}	3.57×10^{1}
50th Percentile	2.60×10^{-1}	2.93×10^{0}

Source: Versar, 1992

5. Soil

The natural habitat for *B. subtilis* is soil. Therefore, long-term survival in soil may be expected to occur. Human exposures via dermal and ingestion routes, and environmental exposures (i.e., to terrestrial, avian, and aquatic organisms via runoff) may occur at the discharge site because of the establishment of *B. subtilis* within the soil.

6. Summary

Although direct monitoring data are unavailable, worst case estimates using sporulation deficient strains do not suggest high levels of exposure to *B. subtilis* to either workers or the public resulting from normal fermentation operations.

V. INTEGRATION OF RISK

A. Discussion

Bacillus subtilis is a ubiquitous, saprophytic, soil bacterium which is thought to contribute to nutrient cycling due to its ability to produce a wide variety of enzymes. This latter feature of the microorganism has been commercially exploited for over a decade. *B. subtilis* has been used for industrial production of proteases, amylases, antibiotics, and specialty chemicals. The Agency has reviewed three submissions for production of enzymes using genetically modified *B. subtilis* and found no unreasonable risks to human health or the environment from the use of this microorganism in fermentation facilities.

Historically, *B. subtilis* was a term given to all aerobic endospore-forming bacilli. Later, *B. subtilis* and two closely related species, *B. licheniformis*, and *B. pumilus*, were grouped taxonomically into what was known as the subtilis-group. However, recently methods have been developed that allow *B. subtilis* to be distinguished from these other species.

B. subtilis is not a frank human pathogen, but has on several occasions been isolated from human infections. Infections attributed to *B. subtilis* include bacteremia, endocarditis, pneumonia, and septicemia. However, these infections were found in patients in compromised immune states. There must be immunosuppression of the host followed by inoculation in high numbers before infection with *B. subtilis* can occur. There also have been several reported cases of food poisoning attributed to large numbers of *B. subtilis* contaminated food. *B. subtilis* does not produce significant quantities of extracellular enzymes or other factors that would predispose it to cause infection. Unlike several other species in the genus, *B. subtilis* is not consider toxigenic. *B. subtilis* does produce the extracellular enzyme subtilisin that has been reported to cause allergic or hypersensitivity reactions in individuals repeatedly exposed to it.

Overall, *B. subtilis* has a low degree of virulence. Although the possibility of human infection is not non-existent, it is low in the industrial setting where exposure to the

bacterium is expected to be low and where highly immunocompromised individuals would not be present. In an industrial setting with the use of proper safety precautions, good laboratory practices, and proper protective clothing and eyewear, the potential for infection of workers should be quite low. The only human health concern for workers in the fermentation facility is the potential for allergic reactions with chronic exposure to subtilisin. As previously stated, OSHA has established an exposure limit to subtilisin which must be met in the industrial setting.

Likewise, the ecological hazards associated with the use of *B. subtilis* are low. There are several reports in the literature on the association of *B. subtilis* with abortions in livestock. However, these few reports indicate that this association must be fairly rare, and typically, the animals were immunocompromised. In addition, *B. subtilis* has not been shown to be a causal agent and is not considered an animal pathogen. Likewise, *B. subtilis* is not considered a plant pathogen. Although it produces enzymes such as polygalacturonase and cellulase that are sometimes associated with the ability to produce soft rot in plant tissue, there are many organisms that are capable of producing a soft rot when injected beneath the outer protective epidermal layers.

The use of *B. subtilis* in an industrial setting should not pose an unreasonable risk to human health or the environment. First, human health and environmental hazards of *B. subtilis* are low. Second, the number of microorganisms released from the fermentation facility is low. In addition, *B. subtilis* is ubiquitous in the environment, and the releases expected from the fermentation facilities will not significantly increase populations of this bacterium in the environment.

In conclusion, the use of *B. subtilis* in fermentation facilities for the production of enzymes or specialty chemicals has low risk. Although not completely innocuous, the industrial use of *B. subtilis* presents low risk of adverse effects to human health or the environment.

B. Recommendations

Asporogenic strains of *B. subtilis* with a sporulation deficiency of at least 10^{-7} are recommended for the tiered exemption. Due to recent changes in classification and the development of new methodology for distinguishing among closely related *Bacillus* species, it is recommended that manufacturers confirm that their strains meet the current classification of *B. subtilis*.

VI. REFERENCES

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